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Circadian Rhythms of Cyanobacteria: Monitoring the Biological Clocks of Individual Colonies by Bioluminescence

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Reproducible circadian rhythms of bioluminescence from individual colonies of cyanobacteria (*Synechococcus* sp. strain PCC 7942) has been observed. Phenotypic monitoring of colonies on agar plates will enable us to genetically analyze the molecular mechanism of the circadian clock of cyanobacteria by screening for clock mutants. By the introduction of a bacterial luciferase gene, we previously developed a transformed cyanobacterial strain (AMC149) that expresses luciferase as a bioluminescent reporter of the circadian clock. In liquid culture, AMC149 expresses a rhythm of bioluminescence that displays the same behavior as circadian rhythms in higher eukaryotes. Improvements in the technique for administering the reporter enzyme's substrate (decanal) and a highly sensitive photon-counting camera allow monitoring the bioluminescence of single colonies. Individual colonies on agar plates displayed a rhythmicity which is essentially the same as that previously reported for liquid cultures.

Circadian rhythms are found in a wide spectrum of organisms and allow them to adapt to a day/night alterations of their environment (3). Physiological studies have revealed that the underlying oscillation (the biological clock) can be generated within single cells (14), but the molecular mechanism of the oscillator is still unknown. Biochemical approaches to discover the clock's mechanism have not been successful to date (6). Molecular genetic approaches are probably the most promising strategy for understanding the oscillator's mechanism, as has been attempted in *Drosophila* (2, 17) and *Neurospora* (13) species. However, in spite of a decade of studies, only two clock genes have been analyzed by molecular cloning (5, 9), and we have not yet obtained a perspective of the structure of the oscillator itself.

As the circadian oscillation that takes ca. 24 h to complete its cycle seems to be composed of many factors, several genes are likely needed to organize the clockwork. Therefore, an experimental model that shows a circadian behavior and can be analyzed by molecular genetics as efficiently as *Escherichia coli* or *Saccharomyces cerevisiae* is essential to dissect the mechanism. We found that a cyanobacterial strain (AMC149), which was constructed by transforming a cyanobacterium (*Synechococcus* sp. strain PCC 7942) with bacterial luciferase genes, displayed a rhythm of bioluminescence. This rhythm fulfills all of the criteria for circadian rhythms as determined for the behavior of eukaryotic organisms (12). The genome of this strain is smaller than that of *E. coli* (10) and much smaller than that of any eukaryotic organism whose clock has been studied. Moreover, this strain is genetically tractable by molecular techniques (8). Thus, it should provide an ideal system for the molecular analysis of the circadian clock.

Our previous measurements of circadian behavior in AMC149 were performed in liquid cultures (12). The ability to monitor circadian rhythms of colonies on agar plates is, however, essential for identifying a clone that expresses a specific phenotype among many colonies to obtain useful mutants and to isolate their complementing genes. To detect the circadian rhythm of individual cyanobacterial colonies, we have monitored bioluminescence of agar plate cultures with a photon-counting camera. The high sensitivity of this camera can detect the bioluminescence of single cyanobacterial colonies. Moreover, we found that signals from individual colonies displayed an evident circadian rhythm of bioluminescence. The rhythms displayed by single colonies are comparable to the rhythms of liquid cultures and fulfill the criteria of circadian behavior. This monitoring capability sets the stage for an aggressive analysis of the circadian mechanism by a genetic approach.

MATERIALS AND METHODS

Cells. We used AMC149, a derivative of *Synechococcus* sp. strain PCC 7942, which was described previously (12). This strain contains the promoter for the *Synechococcus psbAI* gene (7), which encodes the D1 protein of photosystem II, fused to the *Vibrio harveyi* luciferase structural genes (*luxAB* [1]). The reporter gene fusion was recombined into a nonessential region of the *Synechococcus* chromosome (4). A portion of AMC149 culture was spread onto a plate of modified BG-11 agar (4) in a 9-cm-diameter petri dish to yield about 500 colonies per plate. The dish was incubated at 30°C under continuous illumination (LL) of 46 μmol m^{-2} s^{-1} until the colonies were 0.2 to 1.5 mm in diameter (usually 6 to 10 days). In the assay using the photomultiplier tube apparatus, cells are inoculated on the surface of 3 ml of BG-11 agar in a 20-ml vial and cultured similarly. The dish or vial was subjected to darkness for 12 h to synchronize the clocks of the cyanobacterial cells, after which they were returned to LL to have their bioluminescence monitored.

Administration of decanal. Administration of a substrate for bacterial luciferase is crucial for the persistence of a high-amplitude rhythm. We used *n*-decanal, which was purchased from Sigma (St. Louis, Mo.). Decanal was dissolved in vacuum pump oil at a concentration of 3% (vol/vol). A higher concentration was toxic to the cells, and the bioluminescence gradually decays with lower concentrations of decanal. An open sterile pot (a cap of a microcentrifuge tube) containing 0.3 ml of the decanal-oil mixture was placed on the agar surface in the petri dish. The dish was then sealed with Parafilm so that the

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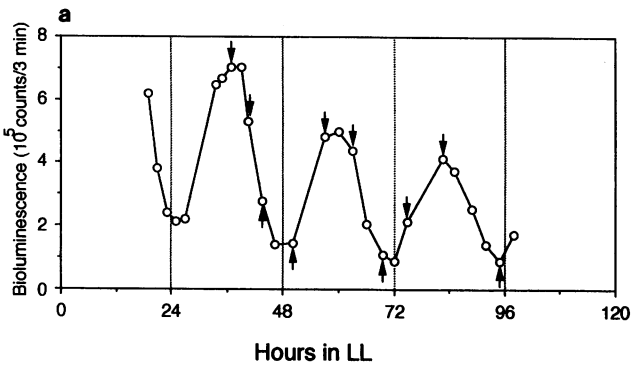
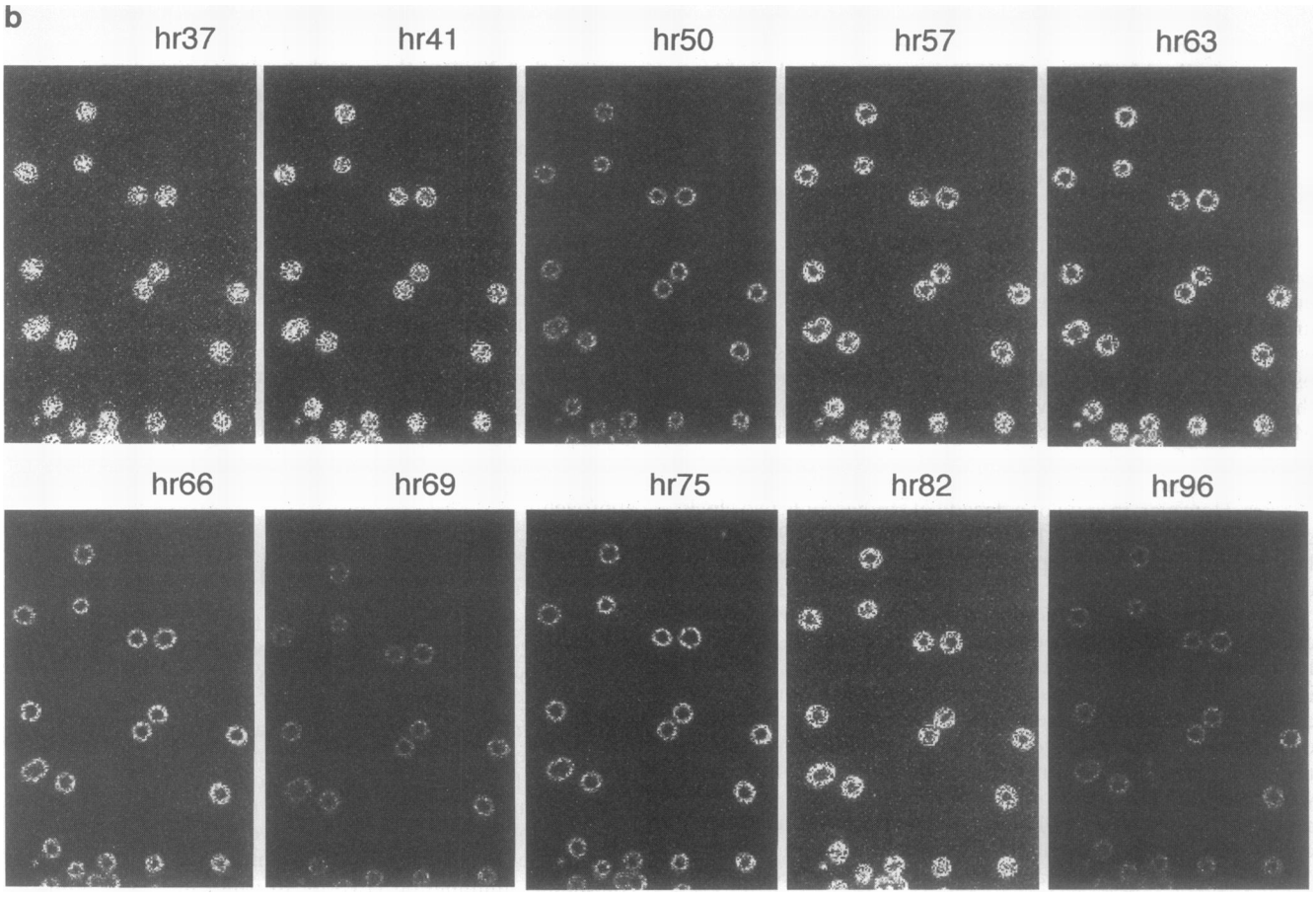


FIG. 1. Rhythmic bioluminescence of an agar plate of AMC149 colonies. Cells were spread on a BG-11 plate and cultured under LL as described in Materials and Methods. Twelve hours of darkness was given to the cells prior to measurement of bioluminescence (i.e., before LL begins). At the indicated time points after release into LL, the plate was subjected to darkness for 6 min: during the last 3 min of this dark period, the bioluminescence image was collected. The time course of bioluminescence measured from the entire plate is shown (Fig. 1a). Bioluminescence images of a rectangular area of 1.5 by 2.7 cm at the time points indicated by arrows are displayed in Fig. 1b.



cells were exposed to the decanal vapor at a constant rate. Bioluminescence of the transformed cells under such conditions reached a stable level within 15 min and continued for longer than a week. For assaying cultures in vials, a sterile microcentrifuge tube containing the decanal-oil mixture (0.3 ml) was placed upright in the vial. The cultures were exposed to decanal vapor from the beginning of the measurement protocol; the phase of the rhythm was not changed by the decanal administration.

Imaging of bioluminescence by a photon-counting camera. A video camera head with an image intensifier installed (512 by 512 pixels; Hamamatsu model C2400-25) was used for imaging bioluminescence of colonies grown on agar. The lens (f = 25 mm) was adjusted to focus on a rectangle inscribed on the petri dish. A system provided by Hamamatsu (ARGUS-100) controlled the operation of camera, data acquisition, and data analysis. The ARGUS-100 image analyzing routine computed the count of the whole field as well as those of regions of interest within the field. Voltage of the multichannel plate and the discrimination threshold for photon counting were automatically adjusted by the system. At various times during LL, the dish was subjected to darkness for 3 min to allow chlorophyll fluorescence to decay, and then the bioluminescence signal was accumulated for the next 3 min. After this 6-min exposure to darkness, the dishes were returned to LL conditions. Temperature was kept constant throughout the experiment, usually at 30°C.

Assay by photomultiplier tube. To measure the period of the rhythm precisely under various temperatures, we used the automated measuring system that was previously described for

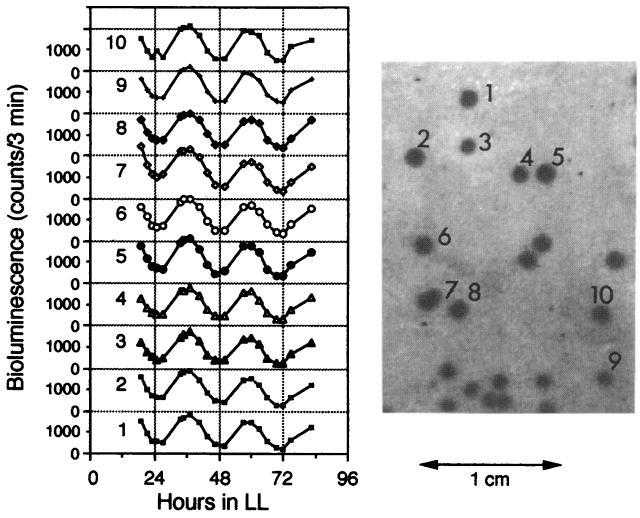


FIG. 2. Circadian rhythms of individual colonies. Time courses of bioluminescence of 10 colonies randomly chosen from the experiment in Fig. 1 are shown. The region used to estimate the bioluminescence of each colony was an oval that encloses the colony, as shown in the photograph. The diameter of each oval was fixed to 18 pixels throughout the experiment.

measuring the rhythms of liquid cultures (12). The design of the apparatus is similar to that of the apparatus used for measuring the bioluminescence rhythms of *Gonyaulax* species (11, 16). Usually measurements were repeated every 30 min for 1 week under LL. The bioluminescence measurements were performed during brief dark exposures as described above for the camera system. The period of the rhythm was computed by linear regression procedure, using the trough positions.

RESULTS AND DISCUSSION

Cells of AMC149 formed colonies of 1.5-mm diameter on BG-11 plates after an incubation of 10 days in LL at 30°C. After exposing the plates to 12 h of darkness to reset the circadian clocks, we monitored the bioluminescence from the plates at various times during LL. Figure 1a shows a time course of the total bioluminescence of a plate. The bioluminescence oscillated in a circadian fashion; i.e., the period was about 24 h. In concert with the changes of total bioluminescence, the bioluminescent images of individual colonies rhythmically changed between bright and dim (Fig. 1b). Note also that the border of the colonies was brighter than the center. Cells at the border are probably more active in metabolism than cells in the center because they can take up more nutrients by virtue of their proximity to fresh media.

We measured the bioluminescence of individual colony by image-analyzing software. Figure 2 depicts the time course of bioluminescence in the regions that surround 10 randomly chosen colonies. All of the regions measured displayed similar rhythms. Note that the data include a thermal dark noise of 100 to 200 counts per 3 min. After subtraction of the dark current, the peak/trough ratio of the rhythm of each region was greater than 5. The result clearly indicates that we can record operation of the circadian clock of individual colonies by imaging with the photon-counting camera.

To confirm that the clock of a colony can be reset as the liquid culture was, 12 h of darkness was given at different times. Figure 3 shows the time course of bioluminescence in LL measured concurrently from two AMC149 plates that had been subjected to 12-h dark periods that were 12 h out of phase. Under LL after the dark period, the bioluminescence from both cultures oscillated with a period of approximately 24 h, but with opposite phases. The peak of bioluminescence occurred at 12 h after the onset of LL and then at 24-h intervals; this phase relationship is the same as that obtained from the liquid culture. These results indicate that the rhythms of colonies were reset by a single dark period, as was the clock in liquid culture. Figure 3 also shows that the rhythm persisted for 8 days. While the level of bioluminescence decreased after 7 days of LL, the rhythmicity was still evident at day 8.

We have examined the temperature compensation of the period, another key characteristic of circadian rhythms, of the rhythm of colonies on agar medium monitored by a photomultiplier tube. The period was approximately constant at different ambient temperatures between 25 and 35°C (23.5 h at 25°C, 23.9 h at 30°C, and 25.6 h at 35°C). The calculated Q_{10} value for frequency of the rhythm was 1.1, which is very close to that obtained for AMC149 liquid culture. Therefore, we can conclude that a circadian clock functions in individual AMC149 colonies as it does in liquid cultures.

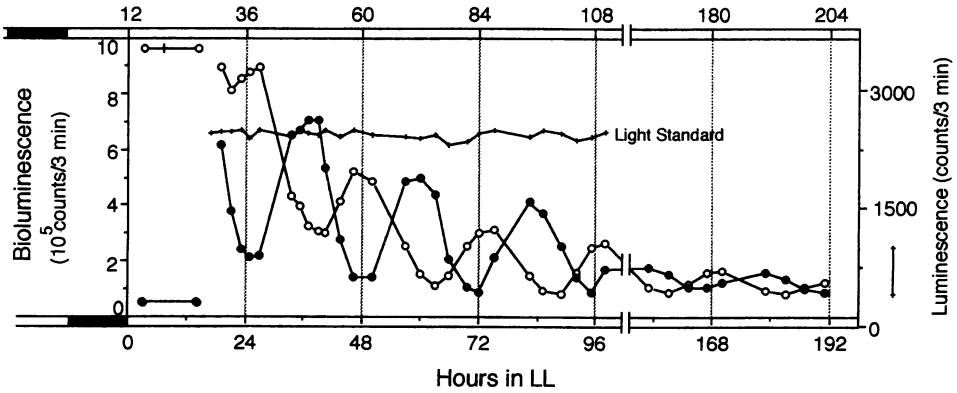


FIG. 3. Resetting of circadian rhythms of bioluminescence by dark periods. The plates of transformed strain AMC149 were cultured at 30°C under LL. The two traces are from cultures that were previously entrained to 12-h dark periods which were 12 h out of phase as illustrated on the abscissa (open bar, light period; closed bar, dark period). The experiment was interrupted at h 99 and then restored at h 150 of the LL. Light standard, 1 μCi of ^{14}C in a scintillation cocktail.

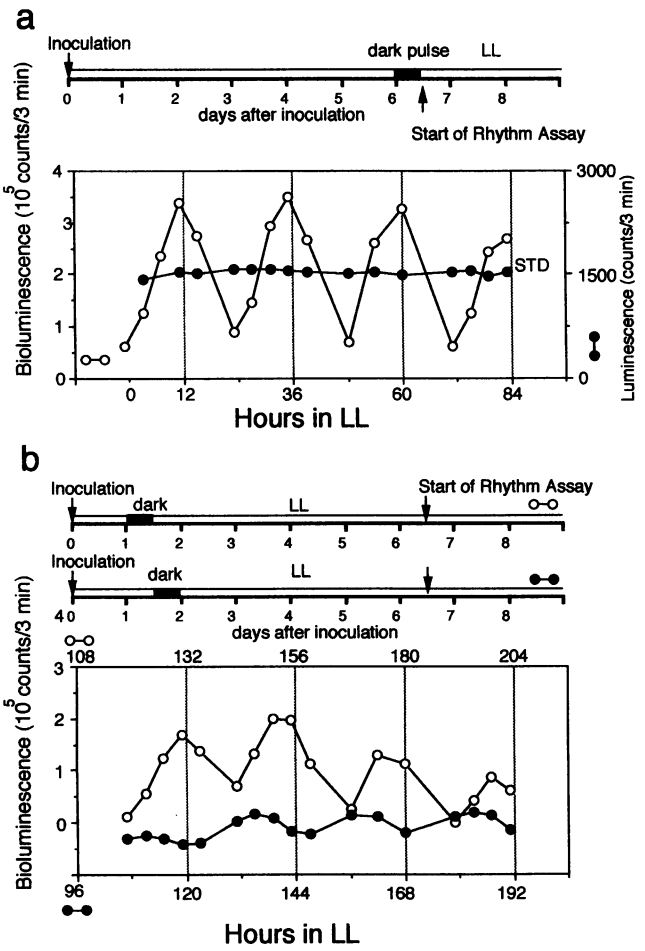


FIG. 4. Bioluminescence rhythms of pinpoint colonies. AMC149 cells (ca. 500 cells) were plated on petri dishes (9-cm diameter) of BG11-agar and incubated under LL. (a) After a 12-h dark period given at day 6, bioluminescence time courses of AMC149 colonies that were 0.1 to 0.2 mm in diameter were measured at various times of LL. (b) After 12-h dark periods given at day 1 (open circles) and day 1.5 (close circles), the cultures were subjected to LL. At day 6.5 (h 120 or 108 of LL), bioluminescence time courses of AMC149 colonies were measured at various times of LL. The left end of the abscissa in panels a and b corresponds to day 6 after inoculation. Data for the light standard as in Fig. 3 are plotted in panel a (STD; closed circles).

Bacterial colonies are not homogeneous; light fluence rate, oxygen/carbon dioxide pressure, and diffusion of nutrients from the medium are all dependent on the exact location of a cell within the colony. As a consequence, the microenvironment of cells within the colony is not the same (15). In our specific case, the availability of decanal to any given cell will also be a function of the location of a cell within the colony. If these factors affect circadian rhythmicity, the circadian behavior would be expected to differ between large colonies (where diffusion could be an important limiting factor) and small colonies (where diffusion should not be as large a problem). Therefore, we checked the rhythm of very young pinpoint colonies (about 0.2 mm in diameter) to examine whether the clock behaves differently in small colonies. Figure 4a shows that the period and phase of the bioluminescence rhythms of pinpoint colonies were essentially the same as those of larger colonies (Fig. 1). Therefore, the factors which could vary between small and large colonies do not appear to have a major impact on the expression of circadian rhythmicity, and we can compare the rhythms of colonies which vary in size.

Furthermore, the clock can be reset at a very early stage of colony formation and the oscillation persists even under the subsequent rapid growth phase. Twelve-hour dark pulses that were 12 h out of phase were given to two plates during the initial stage of colony formation (24 to 36 and 36 to 48 after the initial inoculation). Then, the plates were cultured in LL for 108 or 120 h to form visible colonies. Figure 4b shows that the bioluminescence of these plates oscillated in a circadian fashion and the opposite phases set by the dark pulses were maintained. The cell numbers in a colony were 100 at the time of resetting by the dark pulse and 10^5 at the beginning of rhythm assay (108 or 120 of LL, 0.2-mm-diameter colony). Therefore, the rhythms of these bacteria can persist over at least 10 [$\log_2$ (10^5/100)] cell division cycles in 4 to 5 days.

As the resolution power of the camera is high, we can analyze more clones at the same time by starting with small colonies. Under our culture conditions, the colonies grow slowly, which is advantageous for an assay of the circadian rhythm that usually spans 5 to 7 days. It took 5 days for a 0.5-mm-diameter colony to grow to 1.5 mm in diameter. Therefore, it is practically possible to trace the rhythms of 500 to 1,000 clones from one agar plate, as our cyanobacterial strain is not motile and forms colonies with well-defined edges. The bioluminescence profiles of colonies are more consistent among repeated experiments than the bioluminescence profiles of liquid culture. The physiological conditions of cells and escape of decanal seem to be more stable on plates than in liquid cultures, where agitation is difficult due to the need for an open vial to administer decanal.

We are developing a multiplate system to monitor more than 10 plates concurrently. By using a computer to collect the bioluminescence images, we have determined that it is possible to automatically monitor rhythms of more than 5,000 colonies from 10 plates simultaneously. The images can be analyzed subsequently to identify colonies whose clock properties (e.g., period) are unique. This system will make hunting for clock mutants and screening of clones which have been genetically complemented as efficient as in the studies of nutrition deficiency genes of *E. coli* and will permit saturation mutagenesis for clock-related genes and their analysis.

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